Click-iT® Protein Enrichment Kit

for click chemistry capture of azide-modified proteins

Catalog no. C10416

Table 1. Contents and storage information.

Material	Amount	Storage*
Alkyne agarose resin, 50% slurry (Component A)	2 mL	• 2–6˚C • Do not freeze • Protect from light
Lysis buffer (Component B)	7 mL	• 2–6˚C • Protect from light
Urea (Component C)	4.8 g	• 2–25˚C • Dessicate • Do not freeze
Reaction Additive 1 (Component D)	1.25 mL	• 2–6˚C • Protect from light
Copper (II) sulfate ($CuSO_4$), 100 mM aqueous solution (Component E)	500 µL	≤ 25˚C
Reaction Additive 2 (Component F)	400 mg	
SDS wash buffer (Component G)	200 mL	• 2–6˚C • Do not freeze
Spin columns (Component H)	10 each	≤ 25˚C
Number of assays: Sufficient material is supplied for 10 enrichments, based on the protocol below.		
*When stored as directed, the kit is stable for at least 6 months		

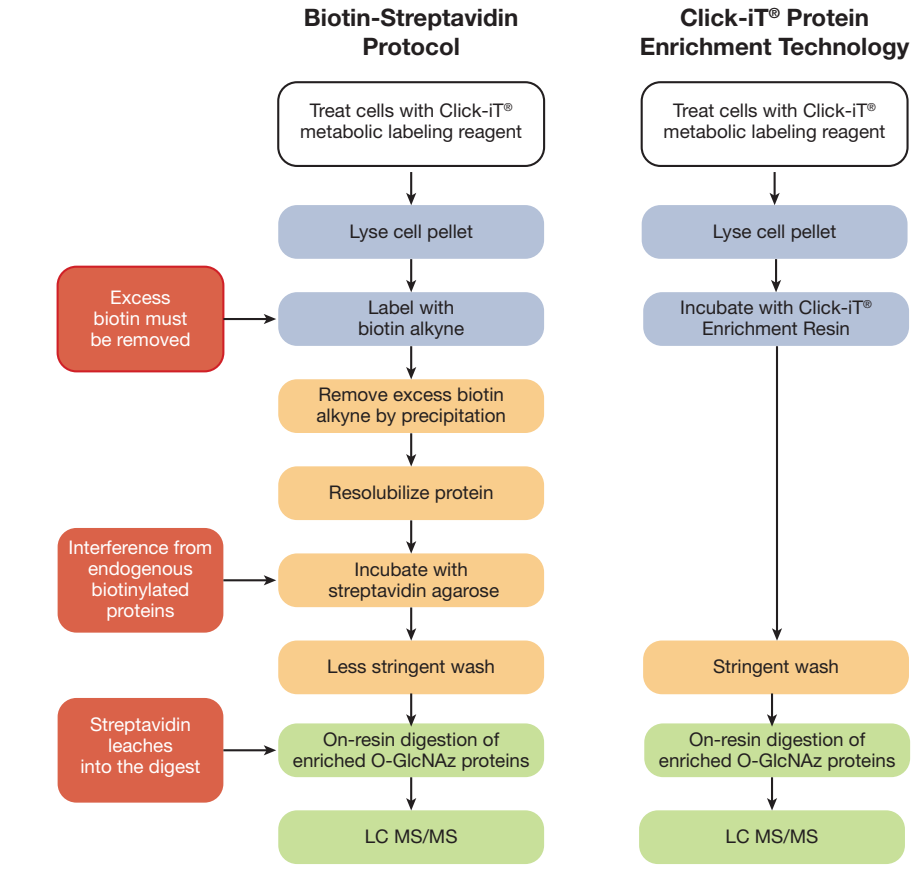
Introduction

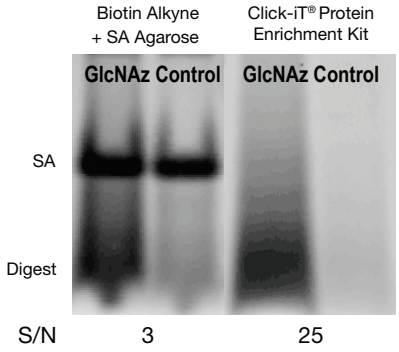
The Click-iT® Protein Enrichment Kit is a tool for enriching specific sub-classes of proteins which have been labeled with an azide tag (see Table 2). The new Click-iT® enrichment resin affords many distinct advantages including:

- Universal differential profiling of expressed and post-translationally modified (PTM) proteins
- Improved signal-to-noise by eliminating nonspecific binding and increasing selectivity, thus improving the detection of low abundant proteins
- Accelerated modification-site identification leading to rapid initiation of functional studies

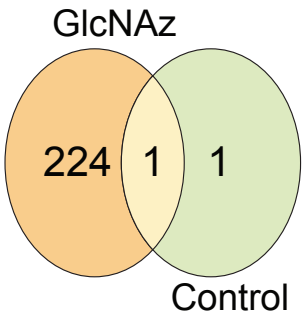
The Click-iT® Protein Enrichment Kit allows efficient covalent capture of newly synthesized proteins and their modified PTM proteins onto an alkyne resin via click chemistry, a Cu(I)

catalyzed cycloaddition reaction between an azide and a terminal alkyne, which generates a 1,4 disubstituted 1,2,3-triazole as a covalent linkage. The alkyne resin containing the covalently coupled PTM proteins can be washed with high stringency, virtually eliminating any non-specifically bound proteins. Upon protease digestion, this yields a highly pure peptide pool that is ideal for mass spectroscopy (e.g., LC MS/MS) based analysis.





SDS-PAGE of GlcNAz and Control digests derived from both methods (SYPRO® Ruby image). A large streptavidin band is visible in the digest from the streptavidin resin.



LC MS/MS analysis of the digests from the Click-iT® Protein Enrichment Kit resulted in identification of 225 proteins in the GlcNAz digest and 2 proteins in the control digest.

Figure 1. The Click-iT® Protein Enrichment Kit is superior to biotin-streptavidin methods. Jurkat cells were treated overnight with 200 µM GlcNAz and lysed. The GlcNAz modified proteins were enriched using either a biotin alkyne and streptavidin agarose protocol or the Click-iT® Protein Enrichment Kit. Challenges for the biotin-streptavidin protocol include the need for (i) a clean-up step to remove the large excess of biotin required in the biotinylation reaction, (ii) the co-enrichment of endogenous biotinylated proteins, (iii) leaching of streptavidin from the resin during the on-resin digestion step, and (iv) loss of some biotinylated proteins during the wash steps. In contrast , the Click-iT® enrichment column demonstrates distinct advantages of covalent binding to the resin, which allows stringent washes without loss of target proteins, thereby enhancing the signal to noise ratio of the data. The purity of Click-iT® Enrichment digest (S/N = 25) is far superior to the biotin-SA digest (S/N = 3) as measured by 280 nm absorbance of the digests.

Table 2. Azide-modified compounds for labeling proteins.

Compound	Application	Cat. no.
Click-iT® GalNAz (tetraacetylated N-azidoacetylgalactosamine)	O-Linked glycoproteins	C33365
Click-iT® GlcNAz (tetraacetylated N-azidoacetylglucosamine)	O-GlcNAc-modified glycoproteins	C33367
Click-iT™ O-GlcNAc Enzymatic Labeling System	O-GlcNAc-modified glycoproteins	C33368
Click-iT® ManNAz (tetraacetylated N-azidoacetyl-D-mannosamine)	Sialic acid-modified proteins	C33366
Click-iT® AHA(L-azidohomoalanine)	Nascent protein synthesis	C10102
Click-iT® palmitic acid, azide	Palmitoylated proteins	C10265
Click-iT® myristic acid, azide	Myristoylated proteins	C10268
Click-iT® farnesyl alcohol, azide	Farnesylated proteins	C10248
Click-iT® geranylgeranyl alcohol, azide	Geranylgeranylated proteins	C10249

Before Starting

Materials Required but Not Provided

- Cell or tissue extract sample (5–20 mg) which has been labeled with an azide-modified biomolecule (Table 2). See **Prepare the Lysate**, next page.
- Unlabeled cells or tissue containing the same relative amount of protein (negative control).
- Protease Inhibitors (e.g., Sigma P8340 or equivalent)
- 2 mL snap-cap polypropylene tubes
- Probe Sonicator or endonuclease such as Benzonase®
- Sample Rotator
- 18 megaOhm water

Note: The following materials are required if you are performing the alkylation, reduction, wash, and on-resin digestion protocols below. You can also substitute other standard reduction, alkylation and digestion methods.

- 1 M DTT, prepared fresh or stored at –20°C
- Iodoacetamide
- 8 M Urea/100 mM Tris, pH 8
- 20% acetonitrile
- 100 mM Tris, pH 8/2 mM $CaCl_2$/10% acetonitrile (Digestion Buffer)
- Mass spectrometry-grade Trypsin, solubilized to 0.1 µg/µL with 50 mM acetic acid, stored –20°C or below
- C-18 desalting cartridges (e.g., Waters WAT036820 or equivalent)
- 0.1% TFA
- 50% acetonitrile/0.1% TFA
- Vacuum concentrator (e.g., SpeedVac or equivalent)

Caution

The Copper (II) sulfate (Component B) is harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment. Avoid release to the environment. Refer to safety data sheets.

Preparing Stock Solutions

Urea Lysis Buffer

1.1 Add the entire contents of Lysis Buffer (Component B) to Urea (Component C) to prepare the Urea Lysis Buffer, consisting of 8 M urea, 200 mM Tris pH 8, 4% CHAPS, 1 M NaCl. Mix on a rocker or rotator at ambient temperature until the urea is completely solubilized, which may take an hour or more. Store at 2–6°C for up to 1 week or at –20°C for up to one year.

1.2 Within 30 minutes of starting the enrichment protocol, supplement the Urea Lysis Buffer with protease inhibitors at twice the concentration recommended by the manufacturer. Prepare 1 mL per enrichment reaction (e.g., if using Sigma P8340, add 20 µL per mL of Urea Lysis Buffer).

Reaction Additive 2

1.3 Add 2 mL of 18 megaOhm water to Reaction Additive 2 (Component F) and mix until fully dissolved. After use, store any remaining stock solution at ≤–20°C for up to 1 year.

Enrichment Protocol

Prepare the Resin

2.1 Thoroughly mix the resin slurry by rocking or rotating the vial until you can see that the resin is completely resuspended.

2.2 Use a 200 µL pipet with 4–5 mm of the tip cut off with a razor blade to pipet 200 µL of slurry into a 2 mL tube.

2.3 Add 1.8 mL of 18 megaOhm water to the resin.

2.4 Pellet the resin by centrifugation for 5 minutes at 1000 rcf. Aspirate the supernatant to waste, leaving approximately 200 µL in the tube and taking care not to aspirate the resin.

Prepare the Lysate

You may perform protein enrichment with azide-labeled cells or tissue extracts and serum. You can lyse the tissues in the provided lysis buffer with protease inhibitors as described for the cells, and you can dilute the serum (100–200 µL) directly into the urea lysis buffer. You may use the lysate prepared from non-azide-labeled cells as a negative control with the azide-labeled cells to evaluate the purity of the enriched proteins.

3.1 Add 850 µL of Urea Lysis Buffer supplemented with protease inhibitors (Step 1.2) to each cell pellet containing 50–200 million cells or to tissue extract containing 5–20 mg of protein.

3.2 Place the lysis mixture on ice for 5–10 minutes, then sonicate it with a probe sonicator, taking care not to overheat the sample.

3.3 Repeat sonication with intermittent cooling until the lysates are no longer viscous and have a water-like consistency (about six 3 second pulses, incubating the lysis mixture on ice for a minute after first three pulses).

3.4 Centrifuge the lysate for 5 minutes at 10,000 rcf. Place the tubes containing the lysate on ice while preparing the catalyst solution.

Prepare the 2X Catalyst Solution

Prepare 1 mL of 2X catalyst solution per enrichment reaction.

4.1 Per 1 mL 2X catalyst solution, combine:

835 µL of 18 megaOhm water
125 µL of Reaction Additive 1 (Component D)
20 µL of 100 mM Copper (II) sulfate (Component E)

4.2 Vortex the solution, then add 20 µL of Reaction Additive 2 (Component F) and vortex again.

Set Up the Click Reaction

5.1 To the 2 mL tube containing the 200 µL of washed resin slurry (from Step 2.4), add:

800 µL of lysate (from Step 3.4; discard the remaining 50 µL containing any insoluble material)
1 mL of 2X catalyst solution (from Step 4.2)

5.2 Rotate end-over-end at room temperature for 18 ± 2 hours.

Reduce and Alkylate the Resin-Bound Protein

6.1 Warm the SDS Wash Buffer to room temperature at least 30 minutes before starting. Invert the bottle and ensure that the solution is homogenous and not cloudy before use.

6.2 Centrifuge the reaction for 1 minute at 1000 rcf. Aspirate the reaction supernatant to waste, taking care not to aspirate the resin.

6.3 Add 1.8 mL of 18 megaOhm water to the resin and repeat the centrifugation and aspiration of the supernatant to waste. This step prevents clumping of the resin caused by the interaction of the residual lysis buffer with the SDS Wash Buffer.

6.4 Add 1 mL of SDS Wash Buffer and 10 µL of 1M DTT to the resin. Vortex briefly to resuspend the resin.

6.5 Heat the resin at 70°C on a heating block for 15 minutes, and then cool at room temperature for 15 minutes.

6.6 Centrifuge the resin for 5 minutes at 1000 rcf, then aspirate the supernatant to waste.

6.7 Prepare 1 mL of iodoacetamine solution per enrichment reaction by dissolving 7.4 mg of iodoacetamide in 1 mL of SDS Wash Buffer.

6.8 Add 1 mL of the iodoacetamine solution to the resin. Vortex briefly to resuspend the resin, and then incubate it in the dark for 30 minutes.

Stringent Washing of the Resin

The SDS Wash Buffer (Component G) provided with the kit allows stringent washing of the resin. Subsequent washing of the resin with 8 M urea and 20% acetonitrile (i.e., multi-mode washing) achieves additional stringent removal of non-specifically bound proteins and SDS prior to on-resin digestion and mass spectrometry analysis of the enriched proteins.

7.1 Snap off the outlet closure from a Spin Column (Component H) and place the column in a rack. Use a 1 mL pipette with 4–5 mm of the tip cut off with a razor blade to resuspend the resin solution from Step 6.8 and transfer it to the column.

7.2 Rinse the resin tube with 0.5 mL of 18 megaOhm water and add the rinse to the column.

Note: Use gravity or vacuum flow for the following washes.

7.3 Add 2 mL of SDS Wash Buffer to the column and allow it to flow to waste. Repeat 5 times.

7.4 Add 2 mL of 8 M urea/100 mM Tris, pH 8 to the column and allow it to flow to waste. Repeat 5–10 times.

7.5 Add 2 mL of 20% acetonitrile to the column and allow it to flow to waste. Repeat 5–10 times

Digest the Resin-Bound Proteins

8.1 Cap the bottom of the column and add 500 µL of digestion buffer (100 mM Tris, 2 mM $CaCl_2$, 10% acetonitrile; not supplied with the kit) to the resin.

8.2 Use a 1 mL pipette with 4–5 mm of the tip cut off with a razor blade to resuspend the resin and transfer it to a clean tube. Rinse the column with 0.5 mL of digestion buffer and add the rinse to the transferred resin.

8.3 Pellet the resin by centrifugation for 5 minutes at 1000 rcf. Aspirate the supernatant to waste, leaving approximately 200 µL of digestion buffer in the tube with the resin, taking care not to aspirate the resin.

8.4 Add 10 µL of 0.1 µg/µL trypsin to the resin slurry, vortex briefly to mix the slurry, and incubate it at 37°C for 6 hours to overnight.

Desalt the Digest

9.1 Pellet the resin by centrifugation for 5 minutes at 1000 rcf, and then transfer the digest supernatant to a clean tube.

9.2 Add 500 µL of water to the resin. Pellet the resin by centrifugation for 5 minutes at 1000 rcf. Add the rinse supernatant to the digest supernatant from Step 9.1.

9.3 Add additional water to the digest to a final volume of 1 mL to dilute the acetonitrile to 2%.

9.4 Acidify the diluted digest with 2 µL of TFA.

9.5 Desalt the digest on a C-18 cartridge using vacuum or gravity flow, allowing each solution to completely flow through the cartridge before adding the next solution:

- Add 1 mL of 50% acetonitrile/0.1% TFA to the cartridge and discard the effluent.
- Add 1 mL of 0.1% TFA to the cartridge and discard the effluent. Repeat once.
- Add the acidified, diluted digest to the cartridge and discard the effluent.
- Add 1 mL of 0.1% TFA to the cartridge and discard the effluent. Repeat once.
- Place a clean 1.5 mL tube below the cartridge outlet.
- Add 700 µL of 50% acetonitrile/0.1% TFA to the cartridge, collecting the eluted peptides into the 1.5 mL tube.

9.6 Dry the eluate containing the desalted digest in a vacuum concentrator (e.g., SpeedVac) and store it at –20°C until mass spectrometry analysis.

Troubleshooting

Problem	Possible Cause	Solution
Low yield of enriched proteins	Inefficient protein capture or Low abundance of azide protein	Increase the lysate concentration (use more cells) or pre-enrich the proteins, if possible (e.g., soluble lysate, membrane lysate, lectin enrichment, etc). **Note:** You can measure the depetion of azide-labeled protein by labeling the starting lysate and the enrichment reaction supernatant with TAMRA alkyne (C10276 and T10183 are required) for SDS PAGE analysis or biotin alkyne (C10276 and B10185 are required) for blot analysis. The peptide recovery of the digest can be measured by 280 nm absorbance with a micro-volume spectrophotometer such as the NanoDrop spectrophotometer (Thermo Fisher), which requires only 2 µL of sample.
	Inefficient digestion of resin-bound proteins	• Use high quality trypsin • Perform Endo Lys-C digestion prior to trypsin digestion
High background in the unlabeled control	Insufficient washing or Contaminated buffers	• Increase column washes • Use high purity reagents and freshly-prepared filtered buffers • Wear gloves • Ensure that the 2X catalyst solution is prepared as directed
Signal suppression in mass spectrometry analysis	Detergent contamination in the digest	Be sure to wash the resin thoroughly after the SDS wash with buffers such as 8 M urea and 20% acetonitrile, which will remove the SDS.

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
C10416	Click-iT® Protein Enrichment Kit *for click chemistry capture of azide-modified proteins*	1 kit
Azide labeling reagents		
C10102	Click-iT® AHA(L-azidohomoalanine) *for nascent protein synthesis*	5 mg
C10248	Click-iT® farnesyl alcohol, azide *mixed isomers*	1 mg
C10249	Click-iT® geranylgeranyl alcohol, azide *mixed isomers*	1 mg
C10265	Click-iT® palmitic acid, azide (15-azidopentadecanoic acid)	1 mg
C10268	Click-iT® myristic acid, azide (12-azidododecanoic acid)	1 mg
C33365	Click-iT® GalNAz (tetraacetylated N-azidoacetylgalactosamine)	5.2 mg
C33366	Click-iT® ManNAz (tetraacetylated N-azidoacetyl-d-mannosamine)	5.2 mg
C33367	Click-iT® GlcNAz (tetraacetylated N-azidoacetylglucosamine)	5.2 mg
C33368	Click-iT® O-GlcNAc Enzymatic Labeling System	1 kit
Mass spectrometry-grade reagents		
C33373	Click-iT® O-GlcNAc peptide and phosphopeptide LC/MS standards, 5 nmol each	1 kit
C33374	Click-iT® O-GlcNAc peptide LC/MS standard (H-Thr-Ala-Pro-Thr-(O- GlcNAc)Ser-Thr-Ile-Ala- Pro-Gly-OH)	5 nmol
MS10015	Mass spectrometry-grade trypsin, 25 µg per vial	10 vials
15505-035	Ultra Pure™ urea	500 g
15505-050	Ultra Pure™ urea	2 kg
15568-025	Ultra Pure™ 1 M Tris-HCl, pH 8	2 kg
Reagents for labeling azide-modified proteins		
A10196	Qdot® 625 streptavidin conjugate *1 µM solution*	200 µL
B10185	Biotin Alkyne	1 mg
C10276	Click-iT® Protein Reaction Buffer Kit	1 kit
R33200	EZQ® Protein Quantitation Kit	10 vials
S12000	SYPRO® Ruby protein gel stain	1 L
S12001	SYPRO® Ruby protein gel stain	200 mL
T10183	Tetramethylrhodamine (TAMRA) alkyne (5-carboxytetramethylrhodamine, propargylamide) *5-isomer*	0.5 mg
W10132	WesternDot™ 625 Goat Anti-Mouse Western Blot Kit *20 mini-gel blots*	1 kit
W10142	WesternDot™ 625 Goat Anti-Rabbit Western Blot Kit *20 mini-gel blots*	1 kit

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Additional international offices are listed at **www.lifetechnologies.com**

These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

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